Emerging Concepts of Regulation of Angiotensin II Receptors
New Players and Targets for Traditional Receptors

Masaki Mogi, Masaru Iwai, Masatsugu Horiuchi

Abstract—Angiotensin (Ang) II exerts its important physiological functions through 2 distinct receptor subtypes, type 1 (AT₁) and type 2 (AT₂) receptors. Recently, new evidence has accumulated showing the existence of several novel receptor interacting proteins and various angiotensin II receptor activation mechanisms beyond the classical actions of receptors for Ang II. These associated proteins could contribute not only to Ang II receptors’ functions, but also to influencing pathophysiological states. Receptor dimerization of Ang II receptors such as homodimer, heterodimer, and complex formation with other G protein–coupled receptors has also been focused on as a new mechanism of their activation or inactivation. Moreover, ligand-independent receptor activation systems such as mechanical stretch for the AT₁ receptor have also been revealed. These emerging concepts of regulation of Ang II receptors and a new insight into future drug discovery are discussed in this review. (Arterioscler Thromb Vasc Biol. 2007;27:2532-2539.)

Key Words: angiotensin II receptor || angiotensin II type-1 receptor blocker || G protein–coupled receptor || interacting protein || dimerization

Critical Determinants of Functions of Angiotensin II Receptors

Ang II receptors are members of the 7 transmembrane G protein–coupled receptors (GPCR). There is relatively low sequence homology between the AT₁ and AT₂ receptors10; however, the third intracellular loop (IC3) of these receptors plays a pivotal role in signaling activation by both receptors. For example, an AT₁/AT₂ chimeric receptor-study showed that substitution of IC3 in the AT₁ receptor failed to induce AT₁ receptor function via Gq protein coupling,11 and also revealed that IC3 is a critical determinant of the mutually antagonistic AT₁ and AT₂ receptor signaling pathways.12 In the AT₂ receptor, IC3 is also important for its function involving reduction of SHP-1 activity and inhibition of extracellular signal regulated kinase (ERK) activity, and AT₂ receptor-induced apoptosis.13 Although IC3 is well known as one of the most important domains of GPCRs for their interaction with G proteins, no interacting proteins with IC3 of both receptors have been reported to date.

GPCRs interact not only with heterotrimeric G proteins but also with accessory proteins called GPCR interacting proteins (GIPs), including soluble proteins. New methodological analysis (yeast 2-hybrid assay or proteomics) has discovered more than 50 GIPs.14 These proteins mainly associate with the carboxyl (C) terminus of GPCRs and regulate their functions by trafficking, fine-tuning, and signaling modifica-
Emerging Concepts of Angiotensin II Receptor Regulation

1. Angiotensin II receptor-associated proteins
   - AT1 receptor: ATRAP, ARAP1
   - AT2 receptor: ATIP (ATBP50) etc.

2. Dimerization
   - Homodimers: AT1 + AT1, AT1 + AT2
   - Heterodimers: AT1 + AT2, Other Receptors + AT1, or AT2

3. Ligand independent activation
   - AT1 receptor: Mechanical Stretch
   - AT2 receptor: Constitutive Activation by Overexpression

4. Agonistic autoantibodies

GIPs with AT1 Receptor

AT1 receptor-associatesn protein (ATRAP) was cloned by our group using a yeast 2-hybrid screening system. ATRAP has 3 transmembrane domains and interacts with the intracellular C-terminal domain of the AT1 receptor (residues 339 and 359), but it does not interact with the AT2 receptor, m1 muscarinic receptor, bradykinin B2 receptor, endothelin ETB receptor, or β1-adrenergic receptor (Figure 1). A stretch of 20 residues upstream of the last C-terminal domain of the AT1 receptor binds to ATRAP. ATRAP could act as a negative regulator in AT1 receptor-mediated cell proliferation and vascular remodeling at least in part by enhancement of AT1 receptor internalization.15,16 Internalization of GPCR is essential for desensitization, endocytosis, and signal transduction of the receptor. The AT1 receptor is well known to undergo rapid endocytosis and downregulation after agonist binding.17 There are several mechanisms involved in AT1 receptor internalization, such as AT1 receptor interacting proteins, receptor phosphorylation,18 and interaction with caveola.19 Moreover, AT1 receptor endocytosis is β-arrestin- and dynamin-dependent via clathrin-coated vesicles at physiological Ang II concentrations.20

ATRAP is expressed in various tissues such as the aorta, heart, and lung, and especially the kidney,21 and colocalizes with the AT1 receptor in mouse renal tubules.22 It has also been reported that overexpression of ATRAP significantly decreases the surface expression of the AT1 receptor in cardiomyocytes and protein synthesis on Ang II treatment.23 indicating that ATRAP promotes downregulation of the AT1 receptor and further attenuates Ang II–mediated cardiac hypertrophy. We observed that expression of ATRAP is temporally decreased in the injured femoral artery of wild-type mice after polyethylene cuff placement, suggesting that this change in ATRAP level may affect remodeling of the injured artery. To examine the pathophysiological roles of ATRAP, we generated ATRAP-transgenic (Tg) mice. This mouse strain exhibits a decrease in neointimal formation, inflammatory response, and NADPH oxidase activity involving a membrane-associated NADPH oxidase subunit, p22phox, in the injured artery compared with those in wild-type, C57BL/6 mice.21 Moreover, in ATRAP-Tg mice, the increase in heart-to-body weight ratio after treatment with Ang II infusion for 14 days or aortic banding is smaller than in wild-type mice. These results indicate that ATRAP plays an inhibitory role in cardiovascular remodeling via regulation of AT1 receptor signaling.

On the other hand, it was reported that ATRAP interacts with calcium-modulating cyclophilin ligand (CAML) using yeast 2-hybrid and immunoprecipitation analysis, and results in regulation of the calcineurin-nuclear factor of activated T cells (NFAT) pathway.24 The calcineurin-NFAT pathway has been implicated in cell growth, differentiation, and immune function involved in atherosclerosis, and negatively regulated cardiac hypertrophy.25 Therefore, in vascular physiology, the CAML-ATRAP interaction is thought to play an important role in the prevention of Ang II-induced pathophysiological actions.

ARAP

In contrast, Inagami and colleagues demonstrated AT1 receptor-associated protein, ARAP1 (AT1 receptor associated protein 1), by a yeast 2-hybrid screening approach as an interacting protein with the carboxyl terminal region of the AT1a receptor (residues 318 to 359).26 The interacting residue is very close to ATRAP; however, ARAP1 promotes recycling of AT1 receptors to the plasma membrane in HEK-293 cells, indicating a presumable role in concomitant recovery of receptor signal functions.27 Overexpression of ARAP1 increased the receptor number in the plasma membrane after Ang II stimulation, whereas overexpression of ATRAP did not affect AT1 receptor internalization; therefore, the regulatory mechanism of AT1 receptor trafficking may differ between ATRAP and ARAP1. Furthermore, kidney specific ARAP1-transgenic (Tg) mice exhibit hypertension and renal hypertrophy with a decrease in urine volume,28 indicating that ARAP1 may have diverse functions from those of ATRAP1, with an opposite effect on trafficking of AT1 receptors.
Although, both GIPs are expressed in vascular smooth muscle cells (VSMCs), the tissue distribution of these GIPs is slightly different. ATRAP is highly expressed in kidney, testis, aorta, and heart, whereas ARAP1 is mainly expressed in kidney, lung, and liver. Moreover, the molecular weight of ARAP1 (57.2 kDa) is 3-fold larger than that of ATRAP (17.8 kDa). Further investigations of their functional aspects and regulation of their expression are necessary to explore their roles in the pathogenesis of cardiovascular remodeling.

Potential Signaling Mechanisms Regulated by ATRAP and ARAP1, and Other Possible GIPs

Phosphorylation of the AT₁ receptor has been implicated in its internalization and activation. Smith et al reported that agonist-induced phosphorylation of the AT₁ receptor is confined to its C-terminal cytoplasmic tail, and this region is implicated in the mechanisms of receptor internalization and desensitization. Phosphorylation of AT₁ receptor induces binding of phospholipase C gamma (PLCγ) 1 to the AT₁ receptor. This PLCγ1-AT₁ receptor interaction depends on tyrosine 319 phosphorylation in a YIPP motif in the C-terminal intracellular domain of the AT₁ receptor, and results in the formation of Src homology 2 domain-containing tyrosine phosphatase (SHP-2) and Jak2 complex and activation of the Jak-STAT pathway.

The Ang II–induced Jak-STAT pathway has a multifaceted role in mediating VSMC growth, migration, and remodeling. This YIPP motif is located in 319 to 322 residues of the C-terminal part of the AT₁ receptor and its association with Jak-STAT pathway is first described by Ali et al. Moreover, phosphorylation of tyrosine 319 within the YIPP motif of the AT₁ receptor also mediates Ang II–induced transactivation of the epidermal growth factor (EGF) receptor. Recently, Zhai et al demonstrated that transgenic mice with cardiac-specific overexpression of an AT₁ receptor with a mutation in tyrosine 319 which was replaced with phenylalanine (Y319F) failed to exhibit agonist-induced AT₁ receptor internalization, involving Ang II and EGF and associated mechanisms such as ATRAP, ARAP1, and caveolin.

Interaction of GIPs with AT₂ Receptor

ATIP, ATBP50, and MTUS1

Proteins interacting with the AT₂ receptor have been highlighted as factors regulating this unique receptor, because the functions of the AT₂ receptor are still an enigma, at least in part. For example, the effect of AT₂ receptor signaling on cardiac hypertrophy is under debate, suggesting the possibility that AT₂ receptor signaling interacting proteins could play key roles in diverse mechanisms of AT₂ receptor signaling. Accordingly, we have cloned AT₂ receptor-interacting protein (ATIP) as a protein interacting with the C-terminal tail of the AT₂ receptor using a yeast 2-hybrid system, and it has been recently shown to cooperate with the AT₂ receptor to transactivate receptor tyrosine kinases independent of G proteins (Figure 2). In Chinese hamster ovary cells expressing the human AT₂ receptor, ATIP inhibits growth factor–induced ERK2 activation and DNA synthesis, and attenuates insulin receptor autophosphorylation, in the same way as the AT₂ receptor. ATIP was found to be identical to a ubiquitously expressed tumor suppressor protein localized in mitochondria. Therefore, ATIP seems to act as a novel early component of the growth inhibitory signaling cascade of the AT₂ receptor.

Recently, we have reported that in rodent neurons, AT₂ receptor stimulation enhanced neural differentiation via translocation of ATIP into the nucleus, indicating that ATIP may play an important role in tissue differentiation such as vascular remodeling. However, the roles of ATIP in vascular remodeling in vivo are totally unknown. Further investigation is necessary to explore the function of ATIP such as using ATIP-gene-engineered mice. ATIP is also named mitochondrial tumor suppressor gene 1 (MTUS1), for which mutation or copy number variants are found in human malignant tumors. ATIP has 3 major transcripts: ATIP1, ATIP3, and ATIP4. ATIP3 is the major transcript in tissues; however, ATIP1 and ATIP4 are mainly expressed in the brain, indicating that ATIP plays biological roles in not only tumor suppression but also brain function.

In contrast, ATBP50 (AT₂ receptor binding protein of 50 kDa), which is identical to ATIP, is reported by Wruck et al to potentially act as a membrane-associated Golgi protein that dictates delivery of the AT₁ receptor to the cell surface. Knocking down of ATBP50 using small interference RNA...
reduced the cell surface expression of the AT2 receptor by translocation of this receptor from Golgi apparatus and attenuated its antiproliferative effects. Moreover, ATBP is strongly expressed in the uterus and adrenal tissue, in which AT2 receptors are highly expressed. In AT2 receptor-deficient mice, ATBP expression was more markedly reduced. Therefore, it is possible that ATBP expression may be regulated by the AT2 receptor. However, the AT2 receptor is also known as an internalization-deficient receptor17,43; thereby, the effect of ATBP50 on its internalization needs to be discussed in the future.

**SHP-1**

Src homology 2 domain–containing protein-tyrosine phosphatase 1 (SHP-1) is one of the tyrosine phosphatases activated by AT2 receptor stimulation and is differentially phosphorylated. AT2 receptor-induced SHP-1 activity as AT2 receptor-mediated inactivation of MAP kinases was first described by Bedecs et al.44 SHP-1 is reported to play an important role in endothelial antioxidative defense controlled through inhibition of NAD(P)H-oxidase activity by negative regulation of PI3K-dependent Rac1 activation.45 SHP-1 is also a pivotal effector in the signal transduction pathway of the AT2 receptor in fetal vascular smooth muscle cells.46 Moreover, SHP-1 is involved in nitric oxide (NO)-induced ERK1/2 dephosphorylation. Interestingly, AT2 receptor-mediated SHP-1 activation is independent of G protein activation,47 indicating that SHP-1 also acts as an inhibitory factor in pathological vascular remodeling, not exerting the effects of a typical GPCR.47 Therefore, we examined the possible interaction of SHP-1 and ATIP and found that AT2 receptor stimulation increases the formation of ATIP and SHP-1 complex and their translocation into the nucleus and enhances cell differentiation in rat neurons.38 In contrast, Sugano et al recently demonstrated that treatment with small interfering RNA (siRNA) against SHP-1 in acute myocardial ischemia markedly reduced the infarct size48 and accelerated angiogenesis through increased phosphorylation of KDR/Flk-1,49 suggesting that knocking down of SHP-1 has beneficial effects on ischemic disease. However, the involvement of Ang II in these experiments is not clear. More detailed analysis of SHP-1 functions in the pathogenesis of cardiovascular remodeling, especially in relation to AT2 receptor stimulation, need to be elucidated to further examine the possibility that SHP-1 could be a target for Ang II–regulated cardiovascular remodeling.

**MMS2**

Moreover, our recent study demonstrated the association of AT2 receptor stimulation with MMS2 induction in neurons via the interaction between ATIP and SHP-1.38,50 MMS2 is one of the ubiquitin-conjugating enzyme-like proteins, and is reported to play an important role in the ubiquitin-proteasome system (UPS) and DNA repair.31 Although, the function of MMS2 in the vasculature has not been reported, AT2 receptor-induced MMS2 upregulation could play an important role in vascular protection after injury, through the DNA repair system. We observed that MMS2 is expressed in rodent vascular smooth muscle cells (LiJuan Min, Masaki Mogi and MaSatsugu Horiuchi, 2007). The detailed roles of MMS2 upregulated by AT2 receptor-induced ATIP and SHP-1 interaction in vascular remodeling are now under investigation.

**PLZF**

On the other hand, the AT2 receptor has also been shown to interact with a transcription factor, promyelocytic zinc finger protein (PLZF), using yeast 2-hybrid studies with the AT2 receptor C-terminal tail as bait.52 PLZF is most strongly expressed in the heart. After Ang II stimulation, PLZF is activated, translocated from the cytosol to the plasma membrane, and colocalizes with the AT2 receptor, resulting in endocytosis. PLZF translocated into the nucleus and nuclear PLZF bind to a consensus sequence of the phosphatidylinositol-3 kinase p85α subunit (p85α-PI3K) gene followed by upregulation of p70 S6 kinase. In cardiomyocytes, PLZF upregulates protein synthesis and induces cardiac hypertrophy (Figure 2).52 D’Amore et al reported that overexpression of the AT2 receptor using adenovirus transfection promotes ligand-independent constitutive cardiomyocyte hypertrophy.53 These results indicate that the AT2 receptor may induce cardiac hypertrophy. However, AT2 receptor activation is well known to directly oppose the effects mediated by the AT1 receptor that enhance cardiac hypertrophy. Moreover, large clinical trials support that ARB treatment, which causes relative stimulation of the AT2 receptor, could prevent cardiac hypertrophy and heart failure. These apparent contradictory results have been discussed, but a conclusion has not been reached. In normal conditions, the AT2 receptor is expressed at an extremely low level in the normal heart compared with that in other tissues. The possible diverse functions via AT2 receptor stimulation may be attributable to not only the distribution of this receptor but also its expression levels. However, changes in the expression of the AT2 receptor under pathophysiological conditions are not yet defined in detail. Therefore, the role of the AT2 receptor in mediating the effects of AngII in the heart has not been well validated.

The existence of other GIPS associated with the AT2 receptor can be expected, and exploration of these potential GIPS could help us to understand the apparently diverse roles of the AT2 receptor in a more elegant fashion. Moreover, examination of transcriptional control of AT2 receptor expression in each pathophysiological condition in different tissues is also awaited especially in relation to AT1 receptor expression. It is possible that the function of the AT2 receptor could be different when the AT1 receptor is activated simultaneously. In other words, the possible cross-talk of AT1 and AT2 receptors needs to be clarified in more detail.

**Other Newly Reported Regulation of Angiotensin II Receptors**

**Complex Formation of Angiotensin II Receptors and Other GPCRs**

The AT1 and AT2 receptors are known as GPCRs, and GPCRs have traditionally been thought to act as monomers, but recent evidence has revealed that GPCRs may form dimers as part of their normal trafficking and function.54 The Ang II receptor is also reported to form homodimers and het-
Angiotensin II Receptor Activation Without Angiotensin II

Ligand-independent activation of GPCRs has been highlighted especially in the discovery of potential new drug targets. Inverse agonists, which were first observed by Costa and Herz, are known as “agonists with a negative intrinsic activity” and block ligand-independent signal transduction by GPCRs. Inverse agonists could stabilize the inactive conformation of the receptor and drive the equilibrium away from the active conformation. Thus, addition of inverse agonists reduces the constitutive activity of the receptor and inhibits basal activity. The Ang II receptor is reported to be activated via ligand-independent mechanisms. For example, mechanical stress activates the AT1 receptor independently of Ang II. This activation can be inhibited by an inverse agonist of the AT1 receptor; therefore, ARBs can be classified as competitive antagonists and inverse agonists. On the other hand, overexpression of the AT2 receptor in COS1 cells itself enhances apoptosis signaling without Ang II stimulation. Moreover, the constitutively active homo-oligomeric Ang II type 2 receptor induces cell signaling independent of receptor conformation and ligand stimulation. Furthermore, the intracellular third loop domain of the AT1 receptor is closely linked with cellular signaling pathways in the alteration of mitogen-activated protein kinase activity and in growth inhibition without Ang II stimulation. Although the detailed mechanisms of ligand-independent Ang II receptor activation remain to be revealed, these approaches may provide new concepts of cardiovascular remodeling and the local renin-angiotensin system.

Angiotensin II Receptor Antibodies

Recently, agonistic antibodies (AA) that target the AT1 receptor have been developed against the second extracellular AT1 receptor loop in women with preeclampsia and in renal transplant recipients during an episode of rejection. Wallukat et al showed that AT1-AA binds to an amino acid sequence of the second extracellular AT1 receptor loop. AT1-AA also induced extracellular signal-related kinase (ERK-1) activation and reactive oxygen species via NADPH oxidase in a similar fashion to Ang II. The detailed role of AT1-AA in preeclampsia and other severe hypertensive conditions has not yet been elucidated.

Future Aspects of Ang II Receptor Regulation in Treatment of Hypertensive Patients

Finally, we review future possible drug discovery in terms of Ang II receptor regulation, focusing on “activation of physiological function”. Activation of the AT2 receptor is expected to have various beneficial effects on not only the cardiovascular system but also other organ disorders; however, there is no appropriate AT2 receptor-agonist available. CGP42112A, which has been used as an AT2 receptor agonist thus far, also has antagonistic effects at higher concentrations. Therefore, a highly agonistic drug for AT2 receptor activation that is widely available is awaited for elucidation of the roles of the AT2 receptor in the pathogenesis of cardiovascular disease. The results of overexpression studies of the AT2 receptor could be quite different from the physiological function of the receptor, because expression of the AT2 receptor is generally lower in adult normal tissues and the AT2 receptor would be transactivated in response to some stimuli such vascular injury. Moreover, the exact localization of the AT1 and AT2 receptors needs to be clarified in more detail in various pathological conditions, because it is possible that the function of the AT1 receptor is different when the AT1 receptor is activated simultaneously. Ligand-independent receptor activation leads us to consider new strategies for inhibition of actual receptor signaling. GPCR blockers are either inverse agonists or neutral antagonists; however, interestingly, 85% of all GPCR blockers are inverse agonists. ARBs are classified into inverse agonists and competitive antagonists. ARBs with inverse agonistic...
effects may inhibit the mechanical stretch-induced mitogenic response and result in their being more effective in preventing cardiac hypertrophy than ARBs without this effect. Comparison of these ARBs by clinical studies will confirm the possible beneficial roles of inverse agonists in humans in the future.

Receptor modification, phosphorylation, and the existence of autoantibodies have also become a consideration beyond the traditional view of drug discovery. The detailed mechanisms of receptor dimerization and these pathophysiological roles have not yet been well elucidated. After clarifying the Ang II receptor interaction with themselves or other receptors, blockade of other receptors will be targeted for the inactivation or activation of Ang II receptors. Treatment with phosphatase and immunosuppressive therapy may also prevent AT1 receptor activation. However, the specificity of phosphatase for its receptor is still raised as a problem. Therefore, future drugs against downstream targets of Ang II receptor signaling as a more specific drug target are expected for the prevention of Ang II–induced cardiovascular disease.

Receptor trafficking is a well-known physiological phenomenon. Selective receptor blockers can inhibit receptor activation but not receptor trafficking. An increase in recycling of the AT1 receptor may have an influence on the therapeutic benefit of ARB. For example, ATRAP is a physiological negative regulator of AT1 receptor signaling via its internalization. Therefore, activation of ATRAP may be more specific and more physiological for inhibition of AT1 receptor signaling. ATIP is also a more specific downstream target of the AT1 receptor, and leads not only to enhancement of neural differentiation, but also to suppression of tumor progression. Further elucidation of the functional regulation of these Ang II receptor interacting proteins including phosphorylation and dephosphorylation, transcriptional control and finding out possible ligands could be useful for new drug discovery for ameliorating the enhanced tissue renin-angiotensin system.

Conclusion

The “Ang II world” has widely spread beyond its classical actions. With recent advances in other GPCR studies, their receptor functions will be elucidated in more detail. Receptor dimerization, ligand-independent pathways, and the roles of interacting proteins with Ang II receptors, can be newly focused on as targets to develop new, more specific pharmacological agents to regulate the actions of Ang II receptors in hypertensive patients. Analysis of the detailed signaling mechanism of Ang II receptors may also clarify the difference between responders and nonresponders to blockade of RAS in patients with hypertension and lead to personalized medical treatment for cardiovascular disease.

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